REMARKS

STATUS OF THE CLAIMS:

Claims 1 to 40 and 52 are cancelled.

Claim 41 has been amended.

Claims 41 to 51 are pending.

Claims 42 to 51 are withdrawn.

Claim 41 has been amended to delete the phrase "one or more of the following". Support for this amendment may be found on pages 4 to 7 of the instant specification. Applicants assert that these amendments were not made to overcome any issues related to the patentability of this claim and that Applicants right to equivalents of Claim 41 is reserved. No new matter has been added.

Claim 41 was further amended to correct a typographical error by deleting "EphA1,". No new matter has been added.

I. Rejections under 35 U.S.C. § 101

a. The Examiner has maintained the rejection of Claim 41 under 35 U.S.C. § 101 alleging it is unpatentable over Claim 16 of US Application 11/072,175 under for the judicially created doctrine of obviousness-type double patenting as being.

Applicants respectfully disagree with the Examiner's allegation. However, Applicants note that the Examiner's rejection is "provisional", and in accordance with MPEP 804(I)(B), no action is required on behalf of Applicants.

II. Rejections under 35 U.S.C. § 112, first paragraph

The Examiner has maintained the rejection of Claim 41 under 35 U.S.C. § 112, first paragraph. Specifically, the Examiner alleges that

> The elected invention is directed to using the EphA2 gene as a predictor for responsiveness of breast cancer cells to protein tyrosine kinase inhibitors. Therefore, results using the predictor sets of Tables 2, 4, and 5 as well as those described in Applicants' declaration are only relevant in as far as they describe the use of the EphA2 gene as a predictor. It is acknowledged, based on the use of BMS-A, as described in the specification, and PP 1, as described for the rejection of Claim 41 under 35 USC 103(a), that the skilled artisan is enabled for using the EphA2 gene as a predictor for determining which breast cancer cells will be sensitive to inhibitors of Src-class kinases. However, the specification fails to enable the skilled artisan to use the EphA2 gene as a predictor for determining which breast cancer cells will be sensitive to inhibitors of BCR-ABL, PDGF-R, c-Kit, EphA1, or EphA2 kinases, as recited in amended Claim 41.
>
> . . .
>
> For these reasons and those set forth in the prior action, rejection of Claim 41 under 35 U.S.C. 112, first paragraph lack of enablement, is maintained.

Applicants disagree with the Examiner's maintenance of the rejection of Claim 41 under 35 U.S.C. § 112, first paragraph, and assert that the claimed invention is enabled by the teachings of the instant specification. However, in the sole interest of facilitating prosecution, Applicant's have amended Claim 41 to delete the phrase "one or more of the following". Applicants point out that BMS-A is an inhibitor of each one of the protein tyrosine kinases recited in Claim 41 as taught by the specification. For example, the specification teaches that "BMS-A has potent inhibitory activity for a number of protein tyrosine kinases, for example, members of the Src family of protein tyrosine kinases, including Src, Fgr, Fyn, Yes, Blk, Hck, Lck and Lyn, as well as the Bcr-abl, Jak, PDGFR, c-

kit and Eph receptors protein tyrosine kinases" (see the paragraph beginning on line 8, page 30). Accordingly, Applicants believe the Examiner's rejection of Claim 41 under 35 U.S.C. § 112, first paragraph, has been rendered moot in consideration of this amendment and Applicants respectfully request that the Examiner withdraw the same.

III. Rejections under 35 U.S.C. § 112 – First Paragraph

a. The Examiner has maintained the rejection of Claim 41 under 35 U.S.C. § 112, first paragraph alleging:

> The claimed methods are not a "genus of methods" requiring the teaching of a number of "representative species". The claimed invention is merely directed to a method of predicting whether a breast cancer cell is sensitive to a protein tyrosine kinase inhibitor. The specification demonstrates that the predictor set of Table 2, 4, and 5 can predict the sensitivity of breast cancer cells to inhibitors of Src-class kinases as well as inhibitors of BCR-ABL, PDGF-R, c-Kit, EphA1, or EphA2 kinases. The 1.131 Declaration filed September 14, 2006 provides further evidence that said predictor sets are useful for predicting the sensitivity to a [sic] additional protein tyrosine kinase inhibitors.
>
> These arguments are not found to be persuasive for the following reasons. Again, the elected invention is directed to using the EphA2 gene as a predictor for responsiveness of breast cancer cells to protein tyrosine kinase inhibitors. The specification (Table 4) and prior art describe use of the EphA2 gene as a predictor for determining which breast cancer cells will be sensitive to inhibitors of Src-class kinases. However, the specification fails to describe, in a manner that conveys that the inventors had possession of the elected invention, a method for using the EphA2 gene as a predictor for determining which breast cancer cells will be sensitive to inhibitors of BCR-ABL, PDGF-R, c-Kit, EphA1, or EphA2 kinases, as recited in amended Claim 41.
>
> For these reasons and those set forth in the prior action, rejection of Claim 41 under 35 U.S.C. 112, first paragraph lack insufficient written description, is maintained.

Applicants disagree with the Examiner's maintenance of the rejection of Claim 41 under 35 U.S.C. § 112, first paragraph, and assert that the claimed invention provides sufficient description to establish that Applicants were in possession of the claimed invention. However, in the sole interest of facilitating prosecution, Applicant's have amended Claim 41 to delete the phrase "one or more of the following". Applicants point out that BMS-A is an inhibitor of each one of the protein tyrosine kinases recited in Claim 41 as taught by the specification as outlined in Section II, *supra*. Accordingly, Applicants believe the Examiner's rejection of Claim 41 under 35 U.S.C. § 112, first

paragraph, has been rendered moot in consideration of this amendment and Applicants respectfully request that the Examiner withdraw the same.

IV. Rejections under 35 U.S.C. § 103

a. The Examiner has maintained the rejection of Claim 41 under 35 U.S.C. § 103(a) over Kassenbrock et al, 2002 in view of Wang et al, 2002 and further in view of Ogawa et at, 2000. More particularly, the Examiner alleges:

> It is acknowledged that, for a rejection under 35 USC 103(a) to be proper, the combined references must teach or suggest all claim limitation. However, it is not required that any one of the instant references teach a method for predicting whether a breast cancer cell will be resistant or sensitive to a protein tyrosine kinase inhibitor by measuring the expression level of the EphA2 receptor. If any of the instant references did teach said method, this would be a rejection under 35 USC 102.
>
> ...(i) Because this is a rejection under 35 USC 103(a), not 102, it is not necessary for Kassenbrock et al to teach that the effect of PP1 to inhibit Cbl phosphorylation would have an antagonistic effect on EphA2. (ii) It is acknowledged that the teachings of Wang et al are that phosphorylation of Cbl is essential for its association and inhibition of EpbA2. Therefore, inhibition of Cbl phosphorylation by PP 1 would be expected to increase expression of EphA2. Claim 4l recites a method using a kinase inhibitor wherein sensitivity to the inhibitor is reflected in an increased expression of EphA2. Therefore, the teachings of Wang et al are consistent with the instant rejection of Claim 41. (iii) See (ii) above.
>
> ...Since PP1 is an inhibitor of Src-class kinases, said amendment does not overcome the instant rejection.

Applicant's disagree with the Examiner's basis for maintaining the rejection of Claim 41 under 35 U.S.C. § 103(a). However, in the sole interest of facilitating prosecution, Applicant's have amended Claim 41 to delete the phrase "one or more of the following". Applicant's point out to the Examiner that PP1 is known to be only an inhibitor of Lck, Fyn, Zap-70, Jak2, and EGF-R (see Abstract and Table I of Hanke et al., J. Biol. Chem., 271(2):695-701 (1996); a copy of which is provided herewith for the convenience of the Examiner). Accordingly, since PP1 is not known to inhibit the kinases specified in Claim 41, the Examiner's rejection of Claim 41 under 35 U.S.C. § 103(a) has been rendered moot in consideration of this amendment and Applicants respectfully request that the Examiner withdraw the same.

Applicants believe that all of the Examiner's rejections and objections have been overcome and that all of the pending claims before the Examiner are in condition for allowance. An early Office Action to that effect is, therefore, earnestly solicited.

A one-month extension is hereby requested pursuant to 37 CFR §1.136(a). Please charge Deposit Account No. 19-3880 in the name of Bristol-Myers Squibb Company in the amount of $120 for payment of the extension fee.

If any fee is due in connection herewith not already accounted for, please charge such fee to Deposit Account No. 19-3880 of the undersigned. Furthermore, if any extension of time not already accounted for is required, such extension is hereby petitioned for, and it is requested that any fee due for said extension be charged to the above-stated Deposit Account.

Respectfully submitted,

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Discovery of a Novel, Potent, and Src Family-selective Tyrosine Kinase Inhibitor

STUDY OF Lck- AND FynT-DEPENDENT T CELL ACTIVATION*

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Here, we have studied the activity of a novel protein-tyrosine kinase inhibitor that is selective for the Src family of tyrosine kinases. We have focused our study on the effects of this compound on T cell receptor-induced T cell activation, a process dependent on the activity of the Src kinases Lck and FynT. This compound is a nanomolar inhibitor of Lck and FynT, inhibits anti-CD3-induced protein-tyrosine kinase activity in T cells, demonstrates selectivity for Lck and FynT over ZAP-70, and preferentially inhibits T cell receptor-dependent anti-CD3-induced T cell proliferation over non-T cell receptor-dependent phorbol 12-myristate 13-acetate/interleukin-2 (IL-2)-induced T cell proliferation. Interestingly, this compound selectively inhibits the induction of the IL-2 gene, but not the granulocyte-macrophage colony-stimulating factor or IL-2 receptor genes. This compound offers a useful new tool for examining the role of the Lck and FynT tyrosine kinases *versus* ZAP-70 in T cell activation as well as the role of other Src family kinases in receptor function.

Study of the roles of the Src family kinases $p56^{lck}$ and $p59^{fynT}$ in T cell receptor (TcR)[1] function has been hampered by a lack of specific pharmacological inhibitors. Here, we describe and utilize a novel, potent, and Src family-selective small molecule inhibitor to further study the role of these kinases in T cell activation.

The *lck* gene, which encodes a lymphocyte-specific, membrane-associated protein-tyrosine kinase of the nonreceptor type (1, 2), was first identified in retrovirally induced murine T cell leukemias (2–4). Lck contains a unique N-terminal sequence (5) that directs its specific interaction with the cytoplasmic domains of the CD4 and CD8 glycoproteins (6–10). This interaction is required for antigen-specific responses of several different T cell hybridomas (11, 12). Lck has also been reported to associate with the IL-2R β-chain via a distinct interaction (13), although its role in IL-2R function remains unclear. Several reports have demonstrated that Lck plays an important role in both T cell maturation and activation. Loss of Lck expression in the human Jurkat T cell line abrogates its response to anti-TcR antibodies (14), and inactivation of the *lck* gene or overexpression of a dominant negative *lck* transgene in mice leads to an arrest of thymocyte development at a stage prior to the expression of CD4, CD8, and TcR (15, 16).

Whereas the role of Lck in T cell development and function is well established, the role of Fyn is less well defined. Small amounts of Fyn are found associated with the TcR complex following mild detergent extraction of T lymphocytes (17). Furthermore, activation of T cells through the TcR results in increased enzymatic activity of Fyn (18). Additional evidence that Fyn is involved in lymphocyte activation comes from experiments in transgenic animals. Twenty-fold overexpression of FynT in transgenic thymocytes results in enhanced responsiveness to anti-CD3 antibody as measured by the stimulation of tyrosine phosphorylation in whole cells, Ca^{2+} accumulation, and proliferation (19). Furthermore, overexpression of a catalytically inactive form of FynT in the thymocytes of transgenic mice substantially inhibited TcR-mediated T cell activation (19). Finally, gene-knockout mice that lack either $p59^{fynT}$ or $p59^{fynB}$ demonstrate defects in TcR/CD3- or alloantigen-mediated signaling (20, 21), suggesting that Fyn plays a role in TcR-mediated signaling *in vivo*.

Here, we show that unlike previously described protein-tyrosine kinase inhibitors, PP1 inhibits Lck and FynT *in vitro* at concentrations significantly lower than those required to inhibit ZAP-70, JAK2, the EGF-R kinase, and protein kinase A. It inhibits whole cell tyrosine phosphorylation and proliferation in T cells stimulated with anti-CD3 and mitogens. Finally, it selectively inhibits IL-2 gene expression over GM-CSF and IL-2R gene induction in human T cells. Thus, this compound appears to dissect a component of TcR signaling not distinguished by other inhibitors of TcR signaling such as FK506. This compound is a powerful new tool to study the role of Src family protein-tyrosine kinases in lymphocyte function.

EXPERIMENTAL PROCEDURES

Cells and Culture Conditions—Human peripheral blood lymphocytes (PBL) were obtained from healthy donors by gradient separation of whole blood using Histopaque (22) and were cultured in RPMI 1640 medium containing 10% fetal calf serum. Anti-CD3-induced proliferation experiments were carried out using 1×10^5 PBL cells/well in 96-well assay plates (Costar Corp.) that were precoated with 20 μg/ml rabbit anti-mouse antiserum (Jackson Laboratories, Bar Harbor, MA) in PBS for 4 h. PBL were treated with varying concentrations of compound and either anti-CD3 (50 ng/ml; Becton Dickinson) or PMA (10 ng/ml; Sigma) plus PHA (2 μg/ml; Wellcome). Proliferation was assessed by the addition of 1 μCi/well [^{3}H]thymidine (DuPont NEN) at 48 h, followed by harvesting the cells at 72 h using a Skatron harvester. Results are reported as an average IC_{50}, determined from a plot of the percent inhibition of proliferation from media control, derived from separate experiments run in triplicate (see Table II). Influenza-induced T cell proliferation (see Table II, Ag-Sp (which represents specific an-

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[1] The abbreviations used are: TcR, T cell receptor; IL-2, interleukin-2; IL-2R, IL-2 receptor; EGF-R, epidermal growth factor receptor; GM-CSF, granulocyte-macrophage colony-stimulating factor; PBL, peripheral blood lymphocyte(s); PBS, phosphate-buffered saline; PMA, phorbol 12-myristate 13-acetate; PHA, phytohemagglutinin; PAGE, polyacrylamide gel electrophoresis.

tigen)) was assessed by combining 1×10^5 PBL in triplicate with 160 μl of RPMI 1640 medium containing 10% fetal calf serum with 20 μl of either diluted test compound or media alone in a 96-well microtiter plate (Costar Corp.). Antigen (influenza virus vaccine Fluzone, Connaught Laboratories) was prepared by centrifuging and washing (three times) 2 ml of vaccine through a Centricon-3 concentrator (Amicon, Inc.) to remove preservative and diluting the remaining material to 40 ml (1:20). Twenty microliters of antigen was then added to each well, and the plates were incubated for 72 h at 37 °C in 5% CO_2. [^{3}H]Thymidine was then added (0.5 μCi/well), and the plates were incubated for an additional 18 h at 37 °C. Cells were harvested with a 96-well harvester (Tomtec), and the amount of incorporated [^{3}H]thymidine was determined using a Pharmacia Biotech β-plate counter. Concentrations that cause 50% inhibition of proliferation (IC_{50}) were determined from a plot of the percent inhibition of proliferation from media control *versus* concentration of test compound added. Results are presented as the mean IC_{50} from repeated experiments (see Table II). T cell proliferation in the one-way mixed lymphocyte reaction was assessed by combining in triplicate 5×10^4 fresh PBL, 5×10^4 irradiated (5000 rads) pooled stimulator PBL, and diluted test compound in RPMI 1640 medium in each well of a 96-well assay plate. After 18 h of incubation at 37 °C in 5% CO_2, 0.5 μCi of [^{3}H]thymidine was added to each well, and the cells were incubated for another 18 h. The cells were then harvested using a Pharmacia Biotech β-plate system. Percent inhibition was determined by the following equation: % inhibition = 1 − (mean cpm of drug-treated cells/mean cpm of control stimulated cells) × 100. IC_{50} values represent the concentration of drug that caused 50% inhibition of the control response. Results are presented as the mean IC_{50} from repeated triplicate experiments (see Table II). Purified human T cells, used in the whole cell phosphotyrosine analyses, were isolated by the T-Kwik method (One Lambda, Canoga Park, CA). T cell purity, as determined by flow cytometric analysis, was generally >90% $CD3^+$ T cells, with the major contaminant being $CD16^+$ cells (natural killer cells). Jurkat T cells (American Type Culture Collection) were maintained in RPMI 1640 medium, 10% fetal calf serum. All cells were maintained at 37 °C in a humidified 5% CO_2 atmosphere.

Immune Complex Enzyme Assays—The enolase substrate (Sigma) used for measuring Lck and FynT catalytic activity (see Fig. 2 and Table I) was prepared as described (23). The acid-treated enolase was diluted 1:20 with 1 × PBS before aliquoting 100 μl/well into a Nunc 96-well high protein binding assay plate. Assay wells were then aspirated; blocked with 0.5% bovine serum, 1 × PBS for 1 h at 37 °C; and then washed five times with 300 μl of 1 × PBS/well. The source of Lck was either LSTRA cells or Lck expressed in HeLa cells using a vaccinia expression system (8, 24). FynT (a gift of R. Perlmutter, University of Washington, Seattle) was expressed in HeLa cells using the vaccinia system (24, 25). Cells (12.5×10^6/ml) were lysed in lysis buffer (20 mM Tris, pH 8.0, 150 mM NaCl, 0.5% Nonidet P-40, and 23 trypsin inhibitory units/ml aprotinin), and the lysates were clarified by centrifugation at 14,000 cpm for 15 min at 4 °C in an Eppendorf tube. The Lck antibody was produced by immunizing rabbits with a synthetic peptide containing residues 41–54 of the N-terminal domain of Lck. The anti-Fyn antibody was obtained from Upstate Biotechnology, Inc. The clarified lysates were then incubated with the appropriate anti-kinase antibody at 10 μg/ml for 2 h at 4 °C. Protein A-Sepharose beads (Pharmacia Biotech Inc.; prepared as a 50% (w/v) suspension) were added to the antibody/lysate mixture at 250 μl/ml and allowed to incubate for 30 min at 4 °C. The beads were then washed twice in 1 ml of lysis buffer and twice in 1 ml of kinase buffer (25 mM HEPES, 3 mM $MnCl_2$, 5 mM $MgCl_2$, and 100 μM sodium orthovanadate) and resuspended to 50% (w/v) in kinase buffer. Twenty-five microliters of the bead suspension was added to each well of the enolase-coated 96-well high protein binding plate together with an appropriate concentration of compound and [γ-^{32}P]ATP (DuPont NEN; 25 μl/well of a 200 μCi/ml solution in kinase buffer). After incubation for 20 min at 20 °C, 60 μl of boiling 2 × solubilization buffer (26) containing 10 mM ATP was added to the assay wells to terminate the reactions. Thirty microliters of the samples was removed from the wells, boiled for 5 min, and run on a 7.5% SDS-polyacrylamide gel. The gels were subsequently dried and exposed to Kodak X-AR film (see Fig. 2*A*). For quantitation, films were scanned using a Molecular Dynamics laser scanner, and the optical density of the major substrate band, enolase p46, was determined. Concentrations of compound that caused 50% inhibition of enolase phosphorylation (IC_{50}) were determined from a plot of the density *versus* concentration of compound (see Fig. 2*B*). In companion experiments for measuring the activity of compounds against Lck (see Fig. 2*C*), the assay plate was washed with two wash cycles on a Skatron harvester using 50 mM EDTA, 1 mM ATP. Scintillation fluid (100 μl) was then added to the wells, and ^{32}P incorporation was measured using a Pharmacia Biotech micro-β-counter. Concentrations of compound that caused 50% inhibition of enzyme activity (IC_{50}) were determined from a plot of the percent inhibition of enzyme activity *versus* concentration of compound. Since there was good correlation between the gel and plate assays, subsequent repeat experiments for both Lck and FynT were performed using scintillation counting (see Table I). EGF-R activity was measured by immunoprecipitation of EGF-R from A-431 cells obtained from the American Type Culture Collection. Cell lysates were prepared by adding 4 ml of lysis buffer to a T-75 flask that contained a confluent layer of cells. The lysates were clarified by centrifugation as described above and then incubated with 10 μg/ml anti-EGF-R (Upstate Biotechnology, Inc.) for 2 h at 4 °C. Protein A-Sepharose beads were added to the antibody/lysate mixture at 250 μl/ml and allowed to incubate for 30 min at 4 °C. The beads were then washed twice in 1.0 ml of lysis buffer and twice in 1.0 ml of kinase buffer (as described above) and finally resuspended to 50% (w/v) in kinase buffer. To each 1.5-ml assay tube was aliquoted 50 μl of bead suspension, which was then spun for 15 s at 14,000 rpm in an Eppendorf microcentrifuge, and the supernatant was discarded. To the bead pellet was then added 5 μl of the appropriate compound dilution, 5 μl of EGF (Upstate Biotechnology, Inc.) to a final concentration of 100 pM, and 5 μl of a 33 μCi [γ-^{32}P]ATP/ml solution in kinase buffer. After incubation for 20 min at 20 °C, the beads were washed once with 1.0 ml of lysis buffer and once with 1.0 ml of 1 × PBS. To the bead pellet was added 60 μl of boiling 2 × solubilization buffer (26) containing 10 mM ATP. Samples were run on 7.5% SDS-polyacrylamide gels, which were subsequently dried and exposed using BAS-III imaging plates. Labeled EGF-R protein bands were visualized, and ^{32}P incorporation was quantitated using the BAS-2000 BioImaging analyzer (Fuji Medical Systems USA, Stamford, CT). Concentrations of compound that caused 50% inhibition of enzyme activity (IC_{50}) were determined from a plot of the percent inhibition of enzyme activity by different concentrations of compound. Murine JAK2 was produced in baculovirus and supplied as an immune complex bound to protein A-Sepharose beads (Upstate Biotechnology, Inc.). JAK2 beads (2.5 μl) were resuspended in 20 μl of kinase buffer (10 mM HEPES, pH 7.4, 50 mM NaCl, 5 mM $MgCl_2$, 5 mM $MnCl_2$, 0.1 mM Na_3VO_4, 0.25 mCi/ml [γ-^{32}P]ATP) for 10 min at room temperature. The beads were then washed, and JAK2 autophosphorylation was measured by eluting the labeled proteins into SDS-PAGE buffer and was analyzed on a 7.5% polyacrylamide gel. Bands corresponding to JAK2 were quantitated using the Fuji BAS-1000 phosphoimager. IC_{50} values were determined as described above. Full-length ZAP-70 kinase (27–29) was produced using baculovirus expression (30). Lysates from Sf9 cells infected 48 h previously with a human ZAP-70 recombinant virus were prepared as described above for Lck, and a 1:100 dilution was used in a soluble kinase assay. Briefly, kinase activity was quantitated by measuring the incorporation of γ-^{32}P into the substrate p62 (31), using SDS-PAGE to resolve phosphorylated p62 and a phosphoimager to quantitate radioactivity. ZAP-70-specific activity was assessed by subtracting p62 phosphorylation obtained using Sf9 cell lysates infected with nonrecombinant baculovirus. IC_{50} values were determined as described above.

Whole Cell Phosphotyrosine Measurements—Inhibition of anti-CD3-stimulated tyrosine phosphorylation in purified human peripheral blood T cells was measured as follows. All incubations were carried out at 37 °C in an Eppendorf Thermomixer 5436 at a mixing setting of 11. Cells (1×10^6 in 100 μl of RPMI 1640 medium) were incubated for 15 min with drug prior to a 6-min incubation with 1 μg of anti-CD3/ml (anti-leu4, 100 μg/ml; Becton Dickinson). The final volume of the reaction was 115 μl. Reactions were terminated by the addition of 57.5 μl of 3 × solubilization buffer (26) incubated at 100 °C prior to its addition. Samples were mixed, boiled for 5 min, and stored at −70 °C. Western blots of these cell lysates, run on 10% SDS-polyacrylamide gels, were probed with a polyclonal anti-phosphotyrosine antibody, and immune complexes were detected with ^{125}I-labeled protein A (ICN) (26). For quantitation, films were scanned using a Molecular Dynamics laser scanner, and the optical densities of the major substrate band, p70, were quantitated in the presence of anti-CD3 (in the presence and absence of drug). Percent inhibition was calculated as follows: (1 − (p70 optical density units in presence of drug/p70 units in absence of drug)) × 100. IC_{50} equals the concentration of compound at which 50% inhibition was measured.

cDNA Probes—Plasmid DNAs were prepared as described (32). cDNA inserts were isolated by digestion with the appropriate restriction enzymes, preparative agarose gel electrophoresis, electroelution, and passage over a G-50 spin column (33). The cDNA probes were labeled using a DNA labeling kit (Pharmacia Biotech Inc.). The human cDNAs for IL-2, GM-CSF, IL-2Rα, and glyceraldehyde-3-phosphate de-

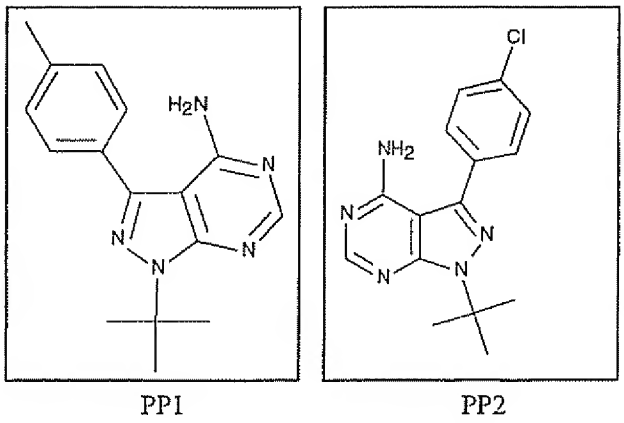

FIG. 1. **Structures of PP1 (4-amino-5-(4-methylphenyl)-7-(*t*-butyl)pyrazolo[3,4-*d*]pyrimidine) and PP2 (4-amino-5-(4-chlorophenyl)-7-(*t*-butyl)pyrazolo[3,4-*d*]pyrimidine).** Both compounds are related to the previously reported pyrrolo[2,3-*d*]pyrimidines (50).

hydrogenase were obtained from the American Type Culture Collection.

Northern Blots—Total RNA was isolated from 1×10^7 fresh isolated human PBL using acid guanidinium/phenol/chloroform (34). Ten to fifteen micrograms of total RNA/lane was electrophoresed on a 6% formaldehyde, 1.5% agarose gel in buffer containing 5 mM $NaHPO_4$, pH 4.45, 15 mM Na_2HPO_4, pH 8.85, and 1.5% formaldehyde. The RNA was capillary-blotted to GeneScreen (DuPont NEN) and UV-cross-linked using a UV Stratalinker 8000. The immobilized RNA was hybridized to probe DNA (4 × 105 dpm/ml) in 50% deionized formamide, 1% SDS, 1 M NaCl, and 10% dextran sulfate. The blots were incubated, with agitation, overnight at 42 °C. The blots were subsequently washed with constant agitation, twice in 2 × SSC (1 × SSC: 0.15 M NaCl, 0.015 M sodium citrate) for 5 min at room temperature, twice for 30 min in 2 × SSC containing 1% SDS at 60 °C, and then twice for 30 min each in 0.1 × SSC at room temperature prior to autoradiography. For each probe, a single blot was stripped and hybridized to cDNA probes specific for each mRNA.

Promoter-Reporter Plasmid Transfections—The promoter-luciferase reporter plasmids were constructed as described previously (32). Briefly, the promoters from the human IL-2 (positions −327 to +51) and IL-2Rα (positions −479 to +109) genes were cloned into pUC13 upstream of the firefly luciferase gene and SV40 polyadenylation signal. The human Jurkat T cell line was transfected as described previously, and luminescence was measured using a Dynatech ML1000 Luminometer. All experiments were run in triplicate, and the data are presented as mean light units. Standard deviations greater than 0.1 are shown.

RESULTS

Selective Inhibition of Src Family Kinases in Vitro—The pyrazolopyrimidine PP1 (Fig. 1) was synthesized as one of a series of compounds used for inhibition of $p56^{lck}$ and $p60^{c\text{-}src}$, based on a parent compound first discovered in tyrosine kinase inhibitor screens. When PP1 was first examined *in vitro* for its ability to inhibit tyrosine phosphorylation of enolase by $p56^{lck}$, it was found to be a potent inhibitor of this enzyme (Fig. 2). $p56^{lck}$ isolated from LSTRA was incubated with enolase and various concentrations of PP1 in a 96-well plate. Solubilization buffer was then added, and the reactions were run out on an SDS-polyacrylamide gel. As shown in Fig. 2*A*, PP1 inhibited phosphorylation of the 46-kDa enolase protein in a dose-dependent manner. The 46-kDa enolase band was quantitated using a Molecular Dynamics laser scanner, and the IC_{50} for inhibition of enolase was ~5 nM (Fig. 2*B*). Duplicate reactions were run in triplicate in the 96-well plate in the presence of various concentrations of PP1 and quantitated using a Pharmacia Biotech micro-β-plate reader (Fig. 2*C*). The IC_{50} for inhibition of Lck in these duplicate reactions using the plate reader was 20 nM. A repeat experiment comparing the gel assay with the plate reader yielded IC_{50} determinations of 8 and 4

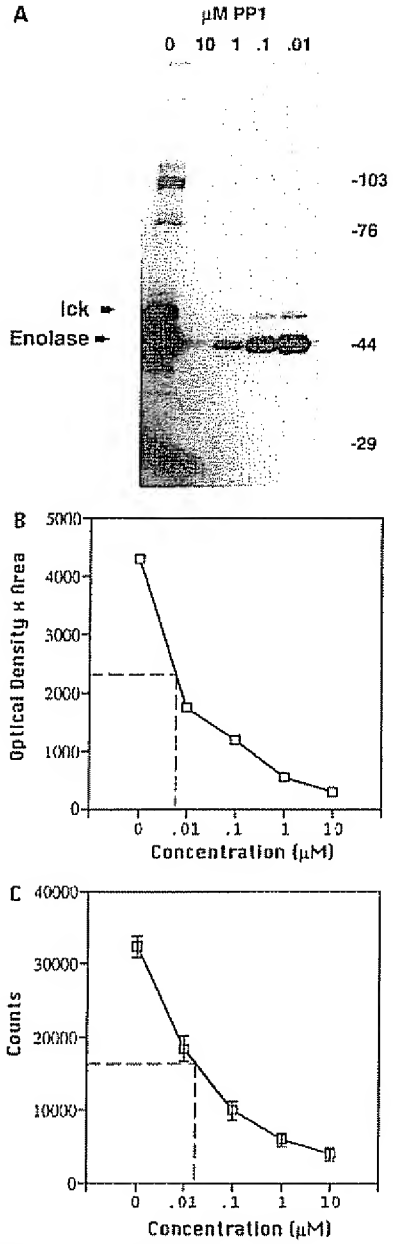

FIG. 2. **Inhibition of Lck catalytic activity *in vitro*.** *A*, Lck phosphorylation of the enolase substrate was analyzed in the presence of increasing concentrations of PP1 using SDS-PAGE as described under "Experimental Procedures." Protein standards (in kilodaltons) are shown on the right. *B*, phosphorylation of the enolase substrate in *A* was quantitated using a Molecular Dynamics laser scanner, and the results are plotted for determination of an IC_{50} value as described under "Experimental Procedures." *C*, duplicate reactions run in parallel with *A* were quantitated using scintillation counting as described under "Experimental Procedures," and the results are plotted to determine an IC_{50} value.

nM, respectively (data not shown). Similar results were obtained using a peptide substrate containing the ITAM sequence derived from the human TcR ζ-chain (data not shown). Since the gel and plate assays provided similar results and the plate reader was more facile for quantitating enolase phosphorylation, subsequent repeat experiments were performed using the plate reader to obtain average IC_{50} values for inhibition of Lck and FynT. In three separate subsequent experiments, PP1 inhibited $p56^{lck}$ activity with an average IC_{50} of 5 nM (Table I). Similar results were obtained with $p56^{lck}$ expressed in HeLa

TABLE I

Inhibitor activity using in vitro kinase assays for Lck, FynT, ZAP-70, JAK2, and EGF-R protein-tyrosine kinases

Average IC_{50} values ± S.D. (in micromolar) are shown as derived from separate experiments. The number of experiments run for each average is shown in parentheses.

Compound	Lck	Fyn	ZAP-70	JAK2	EGF-R
	μM	μM	μM	μM	μM
PP1	0.005 ± 0.001 (n = 3)	0.006 ± 0.001 (n = 3)	>100 (n = 2)	>50 (n = 2)	0.25 (n = 1)
PP2	0.004 ± 0.001 (n = 3)	0.005 ± 0.001 (n = 3)	>100 (n = 2)	>50 (n = 2)	0.48 (n = 1)
Staurosporine	0.003 ± 0.001 (n = 3)	0.005 ± 0.001 (n = 3)	0.02 ± 0.001 (n = 2)	0.04 ± 0.03 (n = 3)	3.3 (n = 1)
Genistein	4.5 ± 1.0 (n = 3)	1.0 ± 0.1 (n = 3)	>100 (n = 2)	>50 (n = 2)	14 (n = 1)

cells using a vaccinia virus expression system (data not shown) (8, 24). The compound was also a potent inhibitor of a second lymphocyte Src family kinase, $p59^{fynT}$ (IC_{50} = 6 nM), expressed using the vaccinia system (Table I). A closely related pyrazolopyrimidine, PP2 (Fig. 1), was similarly effective in the inhibition of Lck and FynT (Table I). In further selectivity tests using other Src family protein-tyrosine kinases, PP1 also inhibited Src (170 nM) and Hck (20 nM), while PP2 demonstrated potent inhibition of Hck (5 nM) (data not shown). In contrast, PP1 and PP2 were both 50–100-fold less active in the inhibition of A-431 epidermal growth factor receptor autophosphorylation (IC_{50} = 0.25 and 0.48 μM, respectively). Further specificity for inhibition of Lck and FynT was demonstrated when it was found that PP1 and PP2 were essentially inactive for inhibition of ZAP-70 and JAK2 (Table I) and protein kinase A (data not shown). Since the activity of the ZAP-70 enzyme may be enhanced following phosphorylation at residue 493 by Lck (35), we also examined whether inhibition of ZAP-70 by PP1 was altered when coexpressed in insect cells together with Lck as described previously (35). Although coexpression of ZAP-70 with the catalytic domain of Lck consistently led to a 3–4-fold elevation in the specific activity of ZAP-70 for the p62 substrate, PP1 was still unable to inhibit this enzyme up to concentrations of 100 μM (data not shown). For comparison purposes, we also examined the activity of staurosporine and genistein, two previously described tyrosine kinase inhibitors. The fermentation product, staurosporine, has previously been demonstrated to be a potent but nonselective protein kinase inhibitor (36, 37). In the experiments reported here (Table I), staurosporine was found to be a nanomolar inhibitor of $p56^{lck}$ and $p59^{fynT}$ as well as a low micromolar inhibitor of the EGF-R kinase. However, unlike PP1 and PP2, it was also a potent inhibitor of the ZAP-70 and JAK2 tyrosine kinases. For further comparison, the naturally occurring isoflavone genistein (36) was tested for its ability to inhibit the four tyrosine kinases. As expected, it was the least potent inhibitor (Table I). Thus, relative to other reported tyrosine kinase inhibitors, the novel compounds PP1 and PP2 demonstrated potent and selective inhibition of the Src family kinases, such as $p56^{lck}$ and $p59^{fynT}$.

Repression of Early Signaling Events in Human T Cells—One of the earliest events in TcR/CD3 triggering of T cells is the stimulation of the tyrosine phosphorylation of multiple substrates (38). Previous studies have demonstrated that tyrosine kinase inhibitors are capable of inhibiting TcR-induced T cell activation (39, 40). Therefore, PP1 was tested for its ability to inhibit anti-CD3-stimulated tyrosine phosphorylation in purified human T cells (Fig. 3). Human T cells were left untreated (*lane 1*) or were treated with 1 μg/ml anti-CD3 alone (*lane 2*) or with anti-CD3 after a 15-min preincubation with 1 μM (*lane 3*), 10 μM (*lane 4*), or 100 μM (*lane 5*) PP1. Following termination of the reactions, anti-phosphotyrosine Western blotting was used to measure stimulation of tyrosine phosphorylation (26). A

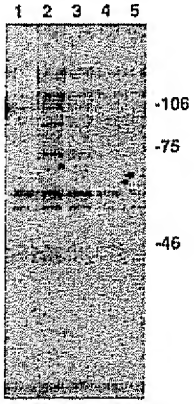

FIG. 3. **PP1 is a potent inhibitor (IC_{50} = 0.55 μM) of T cell tyrosine phosphorylation.** Human peripheral blood T cells were purified as described under "Experimental Procedures." Cells (1×10^6) were incubated for 15 min at 37 °C in either the absence (*lanes 1* and *2*) or presence of a 1 μM (*lane 3*), 10 μM (*lane 4*), or 100 μM (*lane 5*) concentration of the tyrosine kinase inhibitor PP1 prior to the addition of 1 μg of anti-leu4/ml (*lanes 2–5*) or media (*lane 1*). Following an additional 6-min incubation with anti-leu4 (CD3), the reactions were terminated, and the samples were analyzed for tyrosine phosphorylation by Western blotting with polyclonal anti-phosphotyrosine antibody (26) as described under "Experimental Procedures." Protein standards (in kilodaltons) are shown on the right.

representative phosphotyrosine blot is shown in Fig. 3. Anti-CD3 induced the tyrosine phosphorylation of a number of proteins in human T cells (compare *lanes 1* and *2*). These substrates included proteins of approximately 100, 70, 50–60, and 35 kDa. The phosphorylation of all substrates was significantly reduced by 1, 10, and 100 μM PP1 (Fig. 3, *lanes 3–5*, respectively), with 50% inhibition occurring at ~0.5 μM PP1 as measured by quantitating the p70 band from two separate experiments (data not shown). PP2 was equipotent with PP1 for inhibition of anti-CD3-induced tyrosine phosphorylation (data not shown). Thus, treatment of human T cells with this potent Lck and FynT inhibitor blocks the increase in whole cell tyrosine phosphorylation observed following treatment with anti-CD3. These results are consistent with participation of these kinases in early T cell signal transduction; however, they do not rule out a role for other Src family kinases that may be present in T cells.

Inhibition of TcR-induced T Cell Proliferation by PP1—Since PP1 blocked anti-CD3-induced tyrosine phosphorylation, we next examined whether it could inhibit the proliferation of human T cells in response to different stimuli in repeated experiments using different donors (Table II). Human PBL were isolated using Histopaque and plated in the presence of the appropriate compound and stimulus. Proliferation was assessed using [^{3}H]thymidine incorporation. The results are presented as an average of individual experiments performed us-

TABLE II
Inhibition of human PBL proliferation in response to different activating agents

Fresh human PBL were stimulated in the absence or presence of different concentrations of compound. IC_{50} values were determined as described under "Experimental Procedures." Average IC_{50} values ± S.D. are shown as derived from separate experiments. The number of experiments run for each average is shown in parentheses.

Compound	CD3	PMA/IL-2	MLR	Ag-Sp
	μM	μM	μM	μM
Staurosporine	NT[a]	NT	0.003 ± 0.004 (n = 2)	0.4 ± 0.26 (n = 3)
Genistein	11 (n = 1)	3.5 (n = 1)	22 ± 7.3 (n = 3)	>18 (n = 2)
PP1	0.5 ± 0.2 (n = 10)	26 ± 8.8 (n = 7)	3.9 ± 2.8 (n = 5)	5.2 ± 0.98 (n = 4)
PP2	0.6 (n = 1)	18 (n = 1)	1.9 ± 1.5 (n = 4)	4.0 ± 0.3 (n = 3)

[a] NT, not tested.

ing different human PBL donors. An examination of the activity of PP1 yielded an average IC_{50} of 0.5 μM when PBL were treated with anti-CD3 in 10 different donors and 26 μM when proliferation was induced by PMA/IL-2 in seven donors (Table II). Similar results were obtained using purified T cells (data not shown). An examination of other T cell proliferative signals revealed somewhat reduced potency for PP1 (Table II). Fresh human T cells derived from five donors stimulated in a one-way mixed leukocyte reaction were also inhibited with an average IC_{50} of 3.9 μM. T cells derived from healthy donors and stimulated with influenza virus vaccine (Ag-Sp) were inhibited with an average IC_{50} of 5.2 μM (Table II). A similar profile was observed with PP2. Thus, PP1 and PP2 demonstrated inhibition of tyrosine kinase-dependent TcR-induced T cell proliferation. However, they were less effective inhibitors of proliferative signals that by-pass the T cell receptor complex (*e.g.* PMA/IL-2). As a control, staurosporine potently inhibited T cell proliferation in response to both alloantigen (MLR) and specific antigen (Ag-Sp) (Table II), in line with its reported potent broad inhibitory properties (36). Genistein, a previously characterized tyrosine kinase inhibitor (36, 37), demonstrated less potent inhibition of T cell proliferation using all stimuli examined (Table II).

Selective Inhibition of IL-2 Gene Induction by PP1—Since PP1 selectively depressed TcR-dependent proliferation of T cells, we tested whether this compound would demonstrate specificity for inhibition of lymphokine genes involved in T cell proliferation. Human PBL were isolated from a normal donor and were either left untreated (Fig. 4, *lane 1*) or treated with PHA (2 μg/ml) and PMA (10 ng/ml) for 18 h in the absence (*lane 2*) or presence of either 1 μM (*lane 3*) or 5 μM (*lane 4*) PP1. FK506 was used at 0.1 μM as a control (Fig. 4, *lane 5*). IL-2 mRNA induction was almost completely inhibited by both 1 and 5 μM PP1 (Fig. 4, *lanes 3* and *4*, respectively). However, the GM-CSF, IL-2R, and glyceraldehyde-3-phosphate dehydrogenase mRNA levels were not significantly affected by these concentrations of PP1. Interestingly, IL-2R mRNA levels were slightly enhanced in the presence of PP1. In contrast, FK506, a powerful immunosuppressive that is an inhibitor of the Ca^{2+}-dependent phosphatase calcineurin (41), potently suppressed IL-2 and GM-CSF mRNA induction (Fig. 4, *lane 5*), as previously reported (42). IL-2R mRNA induction was also partially repressed by this concentration of FK506. These results suggest that PP1 may selectively inhibit signaling events required for the activation of the IL-2 gene and that the signaling events leading to GM-CSF and IL-2R induction may be distinct.

To further test this hypothesis, we examined whether PP1 could selectively inhibit IL-2 over IL-2R gene expression at the

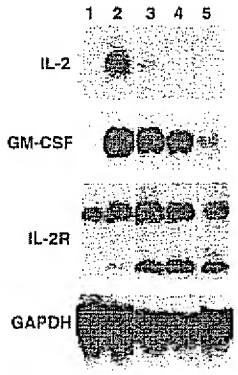

FIG. 4. **Northern analysis of the effects of PP1 on gene expression in human PBL.** Fresh human PBL were either left untreated (*lane 1*) or treated for 18 h in RPMI 1640 medium containing 10% fetal bovine serum with PHA (2 μg/ml) and PMA (10 ng/ml) in the absence (*lane 2*) or presence of 1 μM PP1 (*lane 3*), 5 μM PP1 (*lane 4*), or 0.1 μM FK506 (*lane 5*). Cells were harvested and washed, and total RNA was extracted for Northern analysis as described under "Experimental Procedures." *GAPDH*, glyceraldehyde-3-phosphate dehydrogenase.

level of transcription using reporter plasmids containing either the IL-2 or IL-2R promoter linked to the firefly luciferase gene (Fig. 5). Human Jurkat T cell lines were transfected with these reporter constructs, and the cells were stimulated with PHA (2 μg/ml) and PMA (10 ng/ml) for 18 h prior to assay for luciferase activity as described previously (32). As shown in Fig. 5*A*, both the IL-2 and IL-2R promoter constructs were induced to express luciferase in response to PHA + PMA (compare uninduced (*U*) and stimulated (*S*) *bars* for each reporter construct). PP1 demonstrated dose-dependent inhibition of the IL-2 reporter construct (Fig. 5*B*, *squares*) with an IC_{50} of ~1 μM. In contrast, the IL-2R reporter was not inhibited by PP1 up to concentrations of 35 μM (Fig. 5*B*, *circles*). These results are consistent with the Northern data and suggest that compound PP1 is capable of selectively inhibiting signaling events required for IL-2 gene induction.

DISCUSSION

In this study, we have disclosed the structure and activity of a novel tyrosine kinase inhibitor (PP1) that potently inhibits Lck and FynT, anti-CD3-induced protein tyrosine phosphorylation, and subsequent IL-2 gene activation in T lymphocytes. Moreover, this compound shows selectivity for the Src family over other families of tyrosine kinases including ZAP-70, JAK2, and the EGF-R kinase.

Several properties distinguish PP1 from previously identified tyrosine kinase inhibitors. Its combination of potency (nanomolar *in vitro* and low micromolar in intact cells) and selectivity for members of the Src family of tyrosine kinases is unprecedented. Earlier studies have reported the ability of other tyrosine kinase inhibitors to inhibit signal transduction in T lymphocytes. Prolonged treatment (12–16 h) with 1 μM herbimycin A, a benzoquinoid ansamycin antibiotic, was shown to inhibit TcR-mediated tyrosine phosphorylation, inositol phospholipid hydrolysis, and calcium elevation (40). The lengthy incubation required to observe inhibition and the covalent interaction of herbimycin A with sulfhydryl groups on protein-tyrosine kinases (37) have limited its use. The isoflavone genistein (43) has been also shown to block T cell receptor signal transduction and early activation events (44). In those studies, incubation with 40 μM genistein prior to receptor cross-linking with anti-CD3 significantly inhibited Lck activity and TcR-ζ phosphorylation as well as activation of the IL-2 gene, but was unable to inhibit IL-2 secretion induced by Ca^{2+} ionophore and PMA, agents that by-pass the TcR. The high concen-

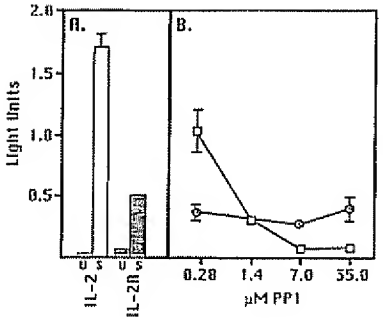

FIG. 5. **Inhibition of IL-2 and IL-2R reporter activity by PP1.** *A*, Jurkat T cells were transfected with either IL-2 or IL-2R promoter-luciferase constructs and then cultured in RPMI 1640 medium containing 10% fetal bovine serum for 18 h in the absence (unstimulated (*U*)) or presence (stimulated (*S*)) of PHA (2 μg/ml) and PMA (10 ng/ml). Cells were then harvested and examined for luciferase activity as described under "Experimental Procedures." *B*, PP1 was added to cells transfected with either the IL-2 (*squares*) or IL-2R (*circles*) reporter constructs for 18 h in the presence of PHA + PMA prior to harvesting the cells and testing for luciferase activity as described under "Experimental Procedures." All values represent the average of three test wells, with standard deviations greater than 0.1 light units shown. The data are representative of numerous repeat experiments.

trations of genistein required to observe inhibition *in vitro* and in whole cells and its lack of specificity in protein kinase inhibition (36) contrast with the potency and specificity we have observed with PP1. A styryl-based protein-tyrosine kinase inhibitor, 67B-83-A (45), was previously shown to be selective for Lck as compared with EGF-R (>100-fold), and it was also selective for inhibition of Lck when compared with other Src family kinases (from >10-fold to >200-fold). However, the compound had an IC_{50} of 7 μM for inhibition of Lck *in vitro*, several orders of magnitude higher than that which we have observed for PP1 using similar protocols for kinase inhibition. Staurosporine, a member of the indolecarbazole group of antibiotics, is also a potent inhibitor of Src family tyrosine kinases *in vitro*. However, in our hands, staurosporine demonstrated less specificity than PP1 and potently inhibited Lck, FynT, ZAP-70, and JAK2 (Table II). Other investigators have similarly reported that staurosporine is a potent inhibitor of Src family kinases (IC_{50} = 90–200 nM) and was also effective for inhibition of nonreceptor tyrosine and serine/threonine protein kinases (36, 37). A new quinolone derivative, WIN 61651, has also been described as an inhibitor of $p56^{lck}$ (46). This compound is significantly less potent than PP1 for inhibition of Lck (18–24 μM) and appears to be less selective since it demonstrates equal potency for the platelet-derived growth factor receptor. Finally, the tyrphostins have been extensively studied for their ability to inhibit various classes of protein-tyrosine kinases (47, 48). Tyrphostins generally show selectivity for protein-tyrosine kinases over other classes of kinases such as protein kinase A, protein kinase C, or Ca^{2+}/calmodulin-dependent kinases. However, less information is available concerning the activities of these compounds on T cell kinases and function. Although studies of tyrosine kinase inhibitors are confounded by the lack of standardized systems for comparison of specificity and potency of protein kinase inhibition, our data support the conclusion that PP1 is the most potent and selective inhibitor of Src family tyrosine kinases such as Lck and FynT reported to date.

The ability of PP1 to dissect signaling pathways was most evident in two of our experiments. First, PP1 was effective at blocking anti-CD3-induced T cell activation events, while it was less effective at inhibiting the TcR-independent proliferation induced by PMA and IL-2. Second, using Northern and reporter assays, we found that PP1 was more effective at inhibiting IL-2 gene expression than either GM-CSF or IL-2R gene induction. These results suggest that Lck and FynT may play a specific role in IL-2 gene expression required for TcR-induced T cell proliferation, but not in the induction of the GM-CSF or IL-2R genes. In contrast, FK506 inhibited anti-CD3-induced expression of IL-2 and GM-CSF mRNAs. Thus, the inhibitory effects of PP1 appear to be more specific than those of FK506. FK506 forms a complex with FKBP, and the FK506-FKBP complex competitively binds to and inhibits the Ca^{2+}/calmodulin-dependent phosphatase calcineurin (41). This results in the inhibition of genes that respond to TcR Ca^{2+}-dependent signaling such as IL-2 and GM-CSF (42). Since PP1 inhibits Ca^{2+} flux in anti-CD3-stimulated T cells (data not shown), it was anticipated that FK506 and PP1 would show similar patterns for inhibition of TcR-induced genes in T cells. However, FK506 and PP1 differentially affected GM-CSF mRNA induction. This suggests that a Ca^{2+}-independent signal may emanate from the TcR to induce GM-CSF expression and that FK506, but not PP1, inhibits this signal. The nature of this other signal and the mechanism by which FK506 may inhibit this other pathway remain to be defined.

The potency of PP1 in inhibiting intracellular activities (*e.g.* T cell tyrosine phosphorylation and proliferation) was considerably reduced relative to inhibition of isolated kinase activity. This difference may be attributed in part to permeability of the compound and its distribution within the cell, but may also be a reflection of the kinetic characteristics of the inhibitor itself. Preliminary studies with PP1 (data not shown) show complex kinetics for inhibition of Lck; however, at certain concentrations of ATP, the compound appears to be competitive with ATP. It therefore is possible that the intracellular millimolar concentrations of ATP found within the cell (49) would act to decrease the potency of the inhibitor *in vivo*. Studies are underway to carefully define the kinetics of Lck inhibition by PP1 and related compounds. Although this property of PP1 and its related compounds appears to limit their usefulness as pharmacological agents in the treatment of T cell-mediated disease, their discovery represents a significant advance in the use of tyrosine kinase inhibitors as tools to study the role of Lck and FynT in T cell signaling.

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